Genetic analysis of selected Mosquito vectors Using Random Amplified Polymorphic DNA (RAPD) Marker in Agra Region

SYNOPSIS

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IN

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By

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INTRODUCTION

Mosquitoes serve as obligate intermediate hosts for numerous diseases that collectively represent a major cause of human mortality and morbidity worldwide. They transmit chronic and debilitating diseases such as malaria, filariasis, yellow fever, chikungunya, Japanese encephalitis, dengue etc. in humans. There are a total of 34 genera and 3100 species of mosquitoes out of which three genera, *Anopheles*, *Aedes* and *Culex*, are the primary vectors for pathogens owing to their obligate haematophagy.

Vector control remains the most successful strategy for the suppression of mosquito-borne diseases. Indiscriminate use of insecticides has resulted in the development of pesticide resistant strains and diminished the effectiveness of biopesticides (Raymond *et al.,* 1991; Roush, 1993). It has been demonstrated in the past that the use of insecticides can dramatically reduce the risk of insect-borne diseases. This is well documented by the WHO and in numerous scientific investigations and reports, particularly concerning the most widespread and important disease, malaria. Once insecticide resistance is established in a population it can profoundly affect public health by the possible reemergence of vector-borne diseases.

Mosquito larvae carry the same resistance genes as adults. Therefore, they are also resistant to the same compounds, although the extent of the resistance might differ between adults and larvae. Since, mosquitoes are found over a large geographical range, they might have evolved intraspecific variations leading to the formation of cryptic species complex posed by the long term usage of insecticides. These cryptic species have often confused epidemiological, ecological and taxonomic research. To facilitate identification of cryptic mosquito species, researchers have employed a wide range of cytological and biochemical approaches, in addition to traditional morphological comparisons. These include analysis of chromosome structure and genetic compatibility

(Foley *et al.,* 1993; Zheng *et al.,* 2005) but the technique cannot be applied to all specimens: it is either sex or developmental stage specific. On the other hand, allozyme electrophoresis requires fresh or frozen material. Some of these techniques lack sufficient resolving power to answer questions at the species or population level, and all are labour and time intensive, requiring laboratory facilities and specialized technical expertise not available to most insect systematists.

Taxonomically, mosquito classification based on above criteria is in a confused state. However, considerable effort has been expanded in finding alternative means of specific identification of mosquitoes, especially to distinguish between sibling species that may differ in their vectorial capacity. Genome organization studies have aided in understanding the systematic and evolution of mosquitoes. These studies are performed by making use of several molecular features such as DNA content, chromosomal, mitochondrial and ribosomal DNA organization, DNA sequences of ITS (internal transcribed spacer) and IGS (inter genic sequences) (Bensansky and Collins, 1992; Hill and Crampton, 1994).

Molecular techniques can be:

- rapid to implement
- performed on multiple samples simultaneously, and
- used to accurately identify individuals to the species level from material of any life stage

Several different methods for documenting genetic information are used. These methods include isozyme analysis, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD) (Mulcahy *et al*., 1993; Hadrys *et al.,* 1992). Although isozyme analysis and RFLPs are a source of readily obtainable genetic information which is easily reproduced, they often do not show polymorphisms which are necessary to determine variation within a group of genetically similar individuals.

With technological advancement, DNA amplification *via* the polymerase chain reaction (PCR) has greatly facilitated DNA sequence comparisons and resulted in the development and use of species diagnostic PCR primer pairs (Paskewitz & Collins, 1990). Even though this is an important application of PCR technology, it still requires

extensive preliminary sequence information for characterization of the taxa under consideration.

Williams *et al*., (1990) and Welsh & McClelland (1990) were the first to describe a novel means of obtaining genetic markers which is not dependent on a priori sequence information, and which may be technically accessible to a wider range of entomologists. This technique, random amplified polymorphic DNA (RAPD) is PCR based, permitting scores of markers to be assayed on DNA extracted from a single mosquito. Instead of using primer pairs as in traditional PCR, RAPD reactions use a single short primer (usually ten bases in length) of randomly chosen sequence. For a RAPD band to be produced, the primer needs to match a binding site that is within approximately 2-3 kilo base pairs of another, oppositely oriented binding site, so that the single oligonucleotide can prime replication in both the forward and reverse direction. A typical RAPD reaction produces multiple amplification products, each representing a discrete genetic locus, which can be analyzed easily by agarose gel electrophoresis.

Additionally, RAPD markers derived from multiple loci and have the potential to provide important information on mosquito population genetic structure that would not be available from a single locus marker. Since its development, RAPD has shown promise for use in a wide variety of organisms including bacteria, higher plants, vertebrates and invertebrates, including mosquitoes and other insects, as a tool for genetic mapping, strain identification and systematics (Williams *et al*., 1993; Chapco *et al.,* 1992; Black *et al*., 1992; Kambhampati *et al*., 1992; Perring *et al*., 1993; Wilkerson *et al*., 1993; Verma *et al*. 2002; Naddaf Dezfouli et al., 2002; Kaur *et al.,* 2007).
RAPD, if used as a complementary tool alongside current morphological identification systems, have the potential to improve the speed and accuracy of various mosquito strains identification which would have evolved in response to the use of mosquito adulticides or larvicides in the recent times.

The study therefore, attempts to identify and estimate genetic variation within mosquito populations (Diptera: Culicidae) focusing on *Anopheles s*p., *Aedes* sp. and *Culex* sp. in Agra region by using PCR based Random Amplified Polymorphic DNA marker technique.

REVIEW OF LITERATURE

The international science literature contains a large number of references to the use of molecular techniques for the identification of insect species (Hill and Crampton, 1994; Sonvico *et al*., 1996; Brown *et al.,* 1997). Many of these references relate to mosquitoes, including phylogenetic studies for taxonomic purposes and for mosquito population research (Severson *et al.,* 1994; Anderson *et al.,* 2001), while others relate to identification of disease-causing agents carried by the mosquito as the vector. Some of these were ELISA based studies for the identification of *Plasmodium falciparum* infected mosquito (Burkot *et al.,* 1984; Wirtz *et al.,* 1987). Chang *et al.* (2001) detected dengue viruses in field caught male *Aedes aegypti* and *Aedes albopictus* by type-specific PCR, whereas, Kramer *et al.* (2002) detected encephalitis viruses in mosquitoes. Recently, a new high-throughput method based on real-time PCR was used for the detection of *Plasmodium* in *Anopheles* mosquitoes (Chris *et al.,* 2008). These methodologies should be amenable for use to establish a biosecurity-targeted strategy for identification of mosquito species.

A large number of the methodologies described in the literature target variability in the ribosomal DNA contained within organisms. Eukaryotic organisms contain both nuclear and mitochondrial ribosomal DNA sequences. These sequences include transcribed genes and intergenic 'spacer' regions (ITS2) between genes. Differing degrees of variability are found with in these regions with the transcribed genes being the least variable while the 'spacer' regions can be highly variable. Variation in these regions can be determined by sequencing, allowing specific probes or primers to be developed to distinguish individuals at various taxonomic levels (Paskewitz *et al*., 1993; Crabtree *et al*., 1995; Debrunner-vossbrinck BA *et al*., 1996; Hackett *et al*., 2000; Krzywinski *et al*., 2001; Shouche and Patole *et al*., 2000; Kampen *et al*., 2005). The mitochondrial cytochrome oxidase I and II genes are another example of highly conserved sequences employed for species level discrimination between insects (Newcomb and Gleeson *et*

al., 1998) and sequence information is available for a number of mosquito species (Morlais *et al.,* 2002; Van Bortel *et al.,* 2002 ; Chen *et al.,* 2002).

Alternative methods that could be employed to identify mosquitoes to species level include, use of microsatellite primers, RAPD primers and RFLP (Restriction Fragment Length Polymorphism). These methods are reported to target highly variable regions of DNA within an organism's genome. Mori *et al*. (1999) reported Comparative linkage maps for the mosquitoes (*Culex pipiens* and *Aedes aegypti*) based on common RFLP loci, whereas Yan *et al.* (1999) studied population genetics of the yellow fever mosquito by using AFLP and RFLP markers. Beebe *et al*. (2002) evaluated species diagnostic polymerase chain reaction-restriction fragment-length polymorphism procedure for cryptic members of the *Culex sitiens*. There are a number of reports which incorporates the use of RAPD-PCR either for the identification of *Anopheles* species or differentiation of its sibling species (Wilkerson *et al.,* 1993; Sucharit and Komalamisra 1997; Sharpe *et al*., 1999; Huong *et al.,* 2001; Naddaf Dezfouli *et al.,* 2002). Studies are also available where randomly amplified polymorphic DNA marker has been used to identify population structure in various *Aedes* and *Culex* species (Davied *et al.*, 1998; Khrabrova et al., 2005).

Extensive literature survey at national level reveals that RAPD markers are recently emerged as a strong tool in the identification of genetic diversity among various species of goats, butterflies, and a number of plant species. (Raghunathachari *et al.,* 2000; Biradar *et al.,* 2005; Nair and Mary 2006; Kaur *et al.,* 2007).

A great deal of work is done in bacterial strain identification using a number of DNA markers (Singh *et al.,* 2002; Verma and Srivastava, 2001). A major work is going on using RAPD, RFLP and MIRU-VNTRs in the identification of *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Katoch *et al*., 2000; Verma *et al*., 2002). However, mosquito borne diseases, which have acquired the status of global threats, there is no report from India where RAPD has been used for their identification.

The proposed work will therefore generate genetic database for the validation of mosquito larvae strains with a quick genetic marker technique i.e. PCR based randomly amplified polymorphic DNA.

OBJECTIVES

1. Standardization of RAPD protocols
2. To assess the diversity of *Anopheles sp., Aedes sp.* and *Culex* by using RAPD markers.
3. To detect genetically distinct clusters of *Anopheles sp., Aedes sp.* and *Culex sp.* in Agra region.
4. Construction of phylogenetic tree on the basis of clusters for *Anopheles sp., Aedes sp.* and *Culex sp.*

METHODOLOGY

STUDY AREA: The study will be carried out in District Agra which is situated in the extreme South-West corner of Uttar Pradesh (27º 10' N and 78º 05' E, a semi-arid zone of Northern India). Various sites will be identified in urban and rural areas including permanent ponds, temporary ponds, irrigated rice fields, road side ditches etc.

LARVAE SAMPLING: Monthly collections will be made for which various sites will be first inspected for the presence of mosquito larvae. Mosquito larvae will be immediately preserved in 95% ethanol. In the laboratory, larvae will be examined microscopically, and separated. Finally, they will be transferred to new vials and preserved in 95% ethanol for subsequent DNA analysis.

SOURCE OF SPECIMENS: Standard mosquito larvae strains for *Anopheles stephensi, Culex quinquefasciatus and Aedes aegypti* will be procured from National Institute Malaria Research (NIMR, New Delhi).

DNA EXTRACTION: Mosquito larvae genomic DNA will be extracted from preserved specimens of IV instar larvae following homogenization of the sample using a pestle grinder according to the DNA extraction protocol from the DNA isolation kits (QIAGEN

DNeasy Blood and Tissue kit). The extracted DNA samples will be stored in TE buffer at -20ºC until further used for PCR amplification.

QUANTIFICATION OF DNA: All the DNA extracted would be subjected to quantification using UV- visible spectrophotometer. Absorbance will be measured at OD_{260} and OD_{280} and the ratio will be calculated.

PCR AMPLIFICATION AND RAPD ANALYSIS: Prior to PCR amplification, DNA samples will be checked for intact DNA on 2 % Agarose gel electrophoresis. For the amplification, readymade PCR kits and Ready to go RAPD kits will be used. A number of primers will be screened to get suitable banding pattern and PCR reaction mixture and annealing temperatures will be standardized. The amplification of all the DNA samples will be repeated three times in order to see the variability, if any, in the amplification patterns. Here is a brief list of universal primers used for the identification of various mosquito species as reported in the literature. The work will be initiated by the screening of following primers:

1) Anopheles sp

a) GGTGACGCAG (Wilkerson *et al.,* 1993; Naddaf Dezfouli *et al.,* 2002)
b) TGGTCAGTGA (Wilkerson *et al.,* 1993)
c) ACACCGATGG (Naddaf Dezfouli *et al.,* 2002)
d) GTAAACCGCC (Naddaf Dezfouli *et al.,* 2002)
e) CCGTCGGTAG (Naddaf Dezfouli *et al.,* 2002)

2) Culex sp

a) TGATCCCTGC (Khrabrova *et al.,* 2005)
b) ATGGTGGAGACGCATGACG (Kasai *et al.,* 2008)
c) GTGGAGACGCATGACGCAT (Kasai *et al.,* 2008)
d) TAGATCCAGACCAGCATCGCG (Sanogo *et al.,* 2007)
e) TAGCRACGAARACCCGTTTGC (Sanogo *et al.,* 2007)

3) Aedes sp

a) GTTGCGATCC (David *et al.,* 1998)
b) GGTCCCTGAC (David *et al.,* 1998)
c) CCGCATCTAC (David *et al.,* 1998)
d) GCGGAAATAG (Ayres *et al.,* 2003)
e) GGTACTCCCA (Ayres *et al.,* 2003)

PHYLOGENETIC ANALYSIS OF THE RAPD PROFILES: RAPD profiles will be used to measure genetic similarity or diversity among various strains of *Anopheles sp., Aedes sp*. and *Culex sp*. in Agra region (we will also sought to differentiate sibling species, if observed, by sequencing specific bands of some selected specimens). Finally the data will be used to generate the Phylogenetic tree with the help of Clustal X or W softwares.

PRELIMINARY WORK DONE:

1. Preliminary literature survey has been done.
2. Collection of mosquito larvae from various sites has been initiated and specimens have been preserved in 95% ethanol for further work.

REFERENCES

Anderson JR, Grimstad PR and Severson DW, 2001. Chromosomal evolution among six mosquito species (Diptera: Culicidae) based on shared restriction fragment length polymorhisms. **Mol Phylogenetic Evol**, 20: 316-321.

Ayres CFJ, Melo-Santos MAV, Sole –Cava AM and Furtado AF, 2003. Genetic Differentiation of *Aedes aegypti* (Diptera: Culicidae), the Major Dengue Vector in Brazil. **J Med Entomol**, 40(4): 430-435.

Black WC IV, Duteau NM, Puterka GJ, Nechols JR, and Pettorini JN, 1992. Use of the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to detect DNA polymorphisms in aphids (Homoptera: Aphididae). **Bull Entomol Res**, 82: 151-159.

Beebe NW, Van Den Hurk AF, Chapman HF, Frances SP, Williams CR and Cooper RD, 2002. Development and evaluation of a species diagnostic polymerase chain reaction-restriction fragment-length polymorphism procedure for cryptic members of the *Culex sitiens* (Diptera: Culicidae) subgroup in Australia and the Southwest. **Pacific J Med Entomology**, 39(2): 362-369.

Biradar DP, Patil VC, Kuruvinashetti MS, Biradar MD, Patil SV and Yaradoni Villa Gualino SN, 2005. The role of biotechnology characterization of bamboo elite clones from Western Ghats of India using RAPD markers. **Turin**, Italy 5-7: 155.

Brown RJ, Malcolm CA, Mason PL and Nichols RA, 1997. Genetic differentiation between and within strains of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) at RAPD loci. **Insect Molecular Biology**, 6: 285-289.

Bensansky NJ and Collins FH, 1992. The mosquito genome Organisation Evolution and Manipulation. **Parasitology Today**, 8: 186–191.

Burkot TR, Williams JL and Schneider I, 1984. Identification of *Plasmodium falciparum*-infected mosquitoes by a double antibody enzyme-linked immunosorbent assay. **Am J Trop Med Hyg**, 33:783-788.

Chen B, Harbach RE and Butlin R K, 2002. Molecular and morphological studies on the *Anopheles minimus* group of mosquitoes in southern China: taxonomic review, distribution and malarial vector status. **Med Vet Entomology**, 16(3): 253-265.

Chung YK, Lim LK and Pang FY, 2001. Detection of Dengue viruses in field caught male *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Singapore by type-specific PCR. **J Med Entomology**, 38(4): 475-479.

Chapco W, Ashton NW, Mattel RKB, and Antonishyn N, 1992. A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers. **Genome,** 35: 569-574.

Chris Bass, Dimitra Nikou, Andrew M Blagborough, John Vontas, Robert E Sinden, Martin S Williamson and Linda M Field, 2008. PCR-based detection of *Plasmodium* in *Anopheles* mosquitoes: a comparison of a new high-throughput assay with existing methods. **Malaria Journal**, 7: 177.

Crabtree MB, Savage HM and Miller BR, 1995 Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. **Am J Trop Med Hyg**, 53: 105–109.

Debrunner-Vossbrinck BA, Vosbrinck CR, Vodkin MH and Novak RJ, 1996. Restriction analysis of the ribosomal DNA internal transcript spacer region of *Culex* restuans mosquitoes in the *Culex pipiens* complex. **J Am Mosq Control Assoc**, 13: 477–482.

David F West and William C and Black IV, 1998. Breeding structure of three snow pool *Aedes* mosquito species in northern Colorado. **Heredity**, 81: 371-380.

Foley DH, Paru R, Dagoro H and Bryan JH, 1993. Allozyme analysis reveals six species within the *Anopheles punctulatus* complex of mosquitoes in Papua New Guinea. **Med Vet Entomol**, 7(1):37-48.

Hackett BJ, Gimnig J, Guelbeogo W, Costantini C, Koekemoer LL, Coetzee M, Collins FH and Besansky NJ, 2000. Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* from *An. rivulorum*, and uncover a cryptic taxon. **Insect Mol Biol**, 9: 369-374.

Hadrys H, Balick M and Schierwater B, 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. **Mol Ecol**, 1: 55-63.

Hill SM and Crampton JM, 1994. DNA-based methods for the identification of insect vectors. **Ann Trop Med and Parasitology**, 88(3): 227-250.

Huong Ngo Thi, Piengchan Sonthayanon, Albert J Ketterman and Sakol Panyim, 2001. A rapid polymerase chain reaction based method for identification of the *Anophelese dirus* sibling species. **J Trop Med Public Health**, 32: 3, 615.

Kambhampati S, Black WC IV and Rai KS, 1992. Random amplified polymorphic DNA of mosquito species and populations (Diptera: Culicidae): Techniques, statistical analysis, nand applications. **J Med Entomol**, 29: 939-945.

Kasai S, Komagata O, Tomita T, Sawabe K, Tsuda Y, Kurahashi H, Ishikaw T, Motoki M, Takahashi T, Tanikawa T, Yoshida M, Shinjo G, Hashimoto T, Higa y and Kabayashi M, 2008. PCR-based identification of culex pipiens complex collected in Japan**. Jpn J Infect Dis**, 61: 184-191.

Katoch VM, Singh D, Chauhan DS, Sharma VD, Singh HB, Das R and Srivastava K, 2000. Newer DNA fingerprinting techniques for tuberculosis-relevance in control. In: *Multi-drug resistance in emerging and re-*

emerging diseases. Mahajan RC, Therwath A (eds.), pp. 87-96, Indian Science Academy & Narosa Publishing House.

Kaur N, Sharma RK, Dhyani D, Karthigeyan S and Ahuja PS, 2007. Molecular characterization of interspecific hybrids of scented roses using RAPD markers. **Acta Hort** (ISHS), 751:175-179.

Khrabrova NV, Sibataev AK and Stegnii VN, 2005. Genetic identification of mosquitoes of the group *Culex pipiens* (Diptera: Culicidae) by RAPD analysis. **Dokl Biochem Biophys**, 401:125-6.

Kramer LD, Wolfe TM, Green EN, Chiles RE, Fallah H, Fang Y and Reisen WK, 2002. Detection of encephalitis viruses in mosquitoes (Diptera: Culicidae) and avian tissues. **J Med Entomology**, 39(2): 312-323.

Krzywinski J, Wilkerson R and Besansky NJ, 2001. Evolution of mitochondrial and ribosomal gene sequences in Anophelinae (Diptera: Culicidae): implications for phylogeny reconstruction. **Mol Phylogenetics Evol**, 18: 479-487.

Kampen H, 2005. The ITS2 ribosomal DNA of *Anopheles beklemishevi* and further remarks on the phylogenetic relationships within the *Anopheles maculipennis* group of species (Diptera: Culicidae). **Parasitol Res**, 97:118-128.

Mori A, Severson DW and Christensen BM, 1999. Comparative linkage maps for the mosquitoes (*Culex pipiens* and *Aedes aegypti*) based on common RFLP loci. **J Hered**, 90(1): 160-644.

Morlais I and Severson DW, 2002. Complete mitochondrial DNA sequence and amino acid analysis of the Cytochrome C Oxidase Subunit I (COI) from *Aedes aegypti*. **DNA Sequence**, 13(2): 123-127.

Mulcahy DL, Cresti M, Sansavini S, Douglas GC, Linskens HF, Bergamini G, Vignani R and Pancaldi M, 1993. The use of random amplified polymorphic DNAs to fingerprint apple genotypes. **Scientia Horticultuae**, 54: 89-96.

Nair NV and Sheji Mary, 2006. RAPD analysis reveals the presence of mainland Indian and Indonesian forms of Erianthus arundinaceus (Retz.) Jeswiet. In the Andaman-Nicobar Islands, India. **Current Science**, 90(8): 1118-1122.

Newcomb RD and Gleeson DM, 1998. Pheromone evolution within the genera Ctenopseustis and Planotortrix (Lepidoptera: Tortricidae) inferred from a phylogeny based on cytochrome oxidase I gene variation - A Laboratory Manual. **Biochemical Systematics and Ecology**, 26(5): 473-484.

Naddaf Dezfouli SR, Oshaghi MA, Vatandoost H, Djavadian E, Telmadarei Z and Assmar M, 2002. Use of Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) and ITS2 PCR assays for

differentiation of populations and putative sibling species of *Anopheles fluviatilis* (Diptera: Culicidae) in Iran. **Iranian J Publ Health**, 31: 3-4, 133-137.

Perring TM, Cooper AD, Rodriguez RJ, Farrar CA and Bellows TS, 1993. Identification of a whitefly species by genomic and behavioral studies. **Science** 259: 74-77.

Paskewitz SM and Collins FH, 1990. Use of the polymerase chain reaction to identify mosquito species of the *Anopheles gambiae* complex. **Med Vet Entomol**, 4: 367-373.

Paskewitz SM, Wesson DM and Collins FH, 1993. The internal transcribed spacers of ribosomal DNA in five members of the *Anopheles gambiae* species complex**. Insect Mol Biol**, 2:247-257.

Raghunathachari P, Khanna VK, Singh US and Singh NK, 2000. RAPD analysis of genetic variability in Indian scented rice germplasm (*Oryza sativa* L.). **Current Science**, 79: 994-998.

Raymond M, Callaghan A, Fort P and Pasteur N, 1991. Worldwide migration of amplified insecticide resistance genes in mosquitoes. **Natrure**, (London) 350: 151–153.

Roush RT, 1993. Occurrence, genetics and management of insecticide resistance. **Parasitology Today**, 9: 174–179.

Severson DW, Mori A, Zhang Y and Christensen BM, 1994. The suitability of restriction fragment length polymorphism markers for evaluating genetic diversity among and synteny between mosquito species. **Am J Trop Med Hyg**, 50(4): 425-432.

Shouche YS and Patole MS, 2000. Sequence analysis of mitochondrial 16S ribosomal RNA gene fragment from seven mosquito species. **Biosci,** 25(4): 361-366.

Singh HB, Chauhan DS, Singh D, Das R, Srivastava K, Yadav VS, Kumar A, Katoch VM and Sharma VD, 2002. Rapid discrimination of Indian isolates of *M. tuberculosis* by random amplified polymorphic DNA (RAPD) analysis – A preliminary report. **Indian J Med Microbiol**, 20: 69-71.

Sonvico Ariane, Manso Fanny and Quesada-Allue Luis A, 1996. Discrimination between the immature stages of *Ceratitis capitata* and *Anastrepha fraterculus* (Diptera: Tephritidae) populations by Random Amplified Polymorphic DNA Polymerase Chain Reaction. **Journal of Economic Entomology**, 89 (5): 1208.

Sucharit S and Komalamisra N, 1997. Differentiation of *Anopheles minimus* species complex by RAPD-PCR technique. **J Med Assoc Thai**, 80 (9): 598-602.

Sharpe RG, Hims MM, Harbach RE and Butlin RK, 1999. PCR-based methods for identification of species of the *Anopheles minimus* group: allele-specific amplification and single-strand conformation polymorphism. **Medical and Veterinary Entomology**, 13: 265-273.

Sanogo YO, Kim CH, Lampman R, and Novak RJ, 2007. A Real-Time TaqMan Polymerase Chain Reaction for the Identification of *Culex* Vectors of West Nile and Saint Louis Encephalitis Viruses in North America. **Am J Trop Med Hyg**, 77(1): 58-66.

Van Bortel W, Sochanta T, Harbach R E, Socheat D, Roelants P, Backeljau T and Coosemans M, 2002. Presence of *Anophales culicifacies* B in Cambodia established by PCR-RFLP assay developed for the identification of *Anopheles minimus* species A and C and four related species. **Med Vet Entomology**, 16(3): 329-334.

Verma R and Srivastava SK, 2001. *Mycobacterium* isolated from man and animals; twelve year record. **Indian J Anim Sci,** 71: 129-132.

Verma R, Singh HB, Sharma VD and Katoch VM, 2002. Molecular characterization of Indian *Mycobacterium bovis* isolates by Random Amplified Polymorphic DNA (RAPD) Analysis – A Preliminary Report. **Indian J Vet Res**, 11: 39-41.

Welsh J and McClelland M, 1990. Fingerprinting genomes using PCR with arbitrary primers. **Nucl Acids Res**, 18: 7213- 721 8.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. **Nucl Acids Res**, 18: 6531-6535.

Williams JGK, Hanafey MK, Rafalski JA and Tingey SV, 1993. Genetic analysis using random amplified polymorphic DNA markers. **Meth Enzymol**, 218: 704-740.

Wilkerson RC, Parson TJ, Albright DG, Klein TA and Braun MJ, 1993. Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae: Anoph). **Insect molecular biology**, 1: 205-211.

Wirtz RA, Zavala F, Charoenvit Y, Campbell GH, Burkot TR, Schneider I, Esser KM, Beaudoin RL and Andre RG, 1987. Comparative testing of monoclonal antibodies against *Plasmodium falciparum* sporozoites for ELISA development. **Bull World Health Organ**, 65:39-45.

Yan G, Romero-Severson J, Walton M, Chadee DD and Severson DW, 1999. Population genetics of the yellow fever mosquito in Trinidad: Comparison of AFLP and RFLP markers. **Mol Ecol**, 8: 951-963.

Zheng B, Tang LH, Ma YJ, Wang XZ, Zhou SS and Shi WQ, 2005. Comparison of PCR and isoenzyme analysis in identification of *Anopheles minimus* A and C. **Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi**, 30: 23(2):78-81.